Telomerase Inhibitors Identified by a Forward Chemical Genetics Approach Using a Yeast Strain with Shortened Telomere Length

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Summary

Telomerase has been proposed as a selective target for cancer chemotherapy. We established a forward chemical genetics approach using a yeast strain with shortened telomere length. Since this strain rapidly enters cell senescence in the absence of active telomerase, compounds that induce selective growth defects against telomere-shortened yeast could be candidates for drugs acting on telomeres and telomerase. We screened our microbial products library and identified three structurally unrelated antibiotics, chrolactomycin, UCS1025A, and radicicol, as active compounds. Detailed analysis showed that chrolactomycin inhibited human telomerase in a cell-free assay as well as in a cellular assay. Long-term culture of cancer cells with chrolactomycin revealed population-doubling-dependent antiproliferative activity accompanied by telomere shortening. These results suggest that chrolactomycin is a telomerase inhibitor, and that the yeast-based assay is useful for discovering the small molecules acting on human telomerase.

Introduction

Telomeres are nucleoprotein structures highly conserved in organisms ranging from yeasts to mammals and are essential for preventing aberrant recombination and protecting against exonucleolytic DNA degradation [1]. Telomerase is a ribonucleoprotein enzyme complex that maintains the telomeric structures at the chromosomal termini by adding the G-rich strand of telomere repeats [2]. Normal human cells either do not express telomerase or express very low levels of it, and, thus, telomeres progressively shorten with each cell division due to incomplete replication of lagging strand DNA synthesis. Loss of telomeres to below a threshold value is believed to induce senescence. In cancer cells, however, telomere length is stably maintained by telomerase, generally at relatively short lengths compared to those in normal somatic cells [3–5]. Thus, telomerase activation is assumed to be the main pathway by which cancer cells become immortal. These properties predict a substantial therapeutic window between cancer and normal cells, and they make telomerase a potentially universal and relatively safe anticancer target for drug discovery.

A number of small-molecule telomerase inhibitors have been reported [6, 7]. Most were identified by random biochemical screening to measure human telomerase inhibitory activity in cell-free systems or by design of modified oligonucleotides, like GRN163 and GRN163L, that target the 11 nucleotide template region of human telomerase RNA (TR) [8, 9]. In some cases, prolonged inhibition of telomerase in various tumor cell lines results in cellular senescence or apoptosis accompanied by telomere shortening.

To identify telomerase inhibitors, we tried a different screening strategy by using a yeast-based cellular assay. We had successfully discovered lead compounds for anticancer therapy by using forward chemical genetics approaches, including Ras farnesyltransferase inhibitors [10], Hsp90 antagonists [11, 12], Src signal transduction inhibitors [13], and proteasome inhibitors [14]. We therefore attempted to develop a new assay system for telomerase inhibitors by applying yeast genetics to drug screening.

We report here the discovery of structurally diversified and unrelated antibiotics, chrolactomycin [15], UCS1025A [16], and radicicol [17], as a new class of telomerase inhibitors identified by a yeast-based assay. Detailed analysis of chrolactomycin showed that this compound inhibited human telomerase in a cell-free assay as well as in a cellular assay. Long-term culture with chrolactomycin revealed population-doubling (PD)-dependent antiproliferative activity accompanied by telomere shortening against human tumor cells. These results suggest that chrolactomycin is an attractive candidate for telomerase inhibition and that the yeast-based assay is useful for the discovery of small molecules acting on human telomerase.

Results

Screening for Telomerase Inhibitors with a Yeast-Based Assay

In budding yeast, mutation of EST1 (*ever shorter telomere 1*), a component of telomerase, leads to progressive telomere shortening and a gradual decrease in growth rate [18]. We produced KY19614 strain, an *EST1* null mutant *S. cerevisiae* harboring the plasmid carrying an *EST1* gene whose expression was under

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the control of a galactose-inducible and glucose-repressive *GAL1* promoter. This strain (KY19614) was inoculated into glucose- or galactose-containing medium and grown for 13 days by diluting the cultures with fresh medium every 24 hr. Southern blot analysis to measure the terminal restriction fragment (TRF) length revealed that this strain exhibited a progressive telomere shortening in glucose medium, indicating a lack of active telomerase in the absence of *EST1* expression (Figure 1).

We established a forward chemical genetics approach by using this budding yeast strain with shortened telomere length, which was produced by conditional deficiency of the *EST1* gene (Figure 2A). The growth of the yeast with critically shortened telomere length depended on the active telomerase regulated by the expression of *EST1*, since this strain could grow in galactose medium, but not in glucose medium. Under conditions in which this strain rapidly entered cell senescence and exhibited a growth-defective phenotype in the absence of active telomerase, compounds that induce selective growth defects in this telomere-shortened yeast strain cultured in galactose medium represent new drug candidates acting on telomeres and telomerase. A parallel screen was conducted by using the telomere-elongated yeast strain to distinguish small molecules that were simply cytotoxic from those acting on telomeres and telomerase.

We selected one of our natural product libraries as a source of screening compounds. Approximately 600 microbial metabolites were screened for their selective activity against telomere-shortened yeast in the paper disc assay. As shown in Figure 2B, 5-fluorouracil (pyrimidine antagonist), nystatin (cell membrane sterol binder), ketoconazole (ergosterol synthesis inhibitor), and wortmannin (phosphatidylinositol 3-kinase inhibitor) were representative nonselective inhibitors detected in this assay, and they yielded inhibition of growth of the two yeast strains equally. Glucose, as a positive control, repressed the *EST1* gene expression and inhibited the growth of the telomere-shortened yeast strain specifically. Although most compounds with anti-yeast activity exhibited nonselective activity against both yeast strains, only three compounds, chrolactomycin, UCS1025A, and radicicol, exhibited selective inhibitory activity against the telomere-shortened yeast strain. Through screening and the detection of reproducible activities, we concluded that these three structurally unrelated antibiotics were active compounds in this yeast-based assay (Figure 2C).

To confirm the principle of the yeast-based assay directing the identification of the compound to regulate telomere length, we investigated the effect on telomere length of yeast treated with one of the active compounds, radicicol. NBW6 strain, a wild-type *S. cerevisiae*, was inoculated into 0, 2, 10, or 50 μM radicicol-containing liquid medium and grown for 27 days by diluting the cultures with fresh radicicol-containing medium every 24 hr. Southern blot analysis to measure TRF length revealed time- and concentration-dependent telomere shortening (Figure S1; see the Supplemental Data available with this article online). This result suggested that the active compounds screened in this assay could actually induce telomere shortening, presumably due to inhibition of telomerase function in yeast.
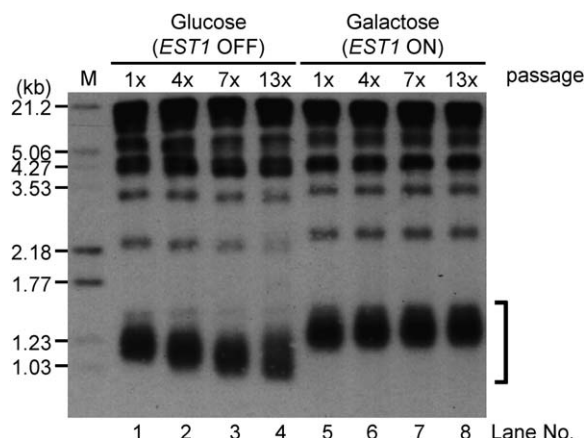

Figure 1. Effect of *EST1* Gene Expression on Telomere Length

The *S. cerevisiae* KY19614 (Δ*EST1 GAL1-EST1*) strain was grown on galactose- or glucose-containing media and was subcultured every 24 hr. Genomic DNA was extracted, digested with *Xho*I, and fractionated by agarose gel electrophoresis. After Southern blotting, telomeric DNA was detected with poly(d[CA/GT]) probe. The bracket indicates the telomeric GT repeat band derived from Y' element-containing chromosomes. Cells were estimated to have undergone between five and seven divisions per passage in liquid medium. The numbering at the top of the lanes (1×, 4×, etc.) indicates the number of times of passage in liquid culture.

Inhibition of Human Telomerase by Screening Hit Compounds in a Cell-free Assay

We were interested in whether the active compounds exhibited inhibitory activity against human telomerase because we would like to assess the potentialities of the active compounds as antitumor agents targeting human telomerase. To examine the effects of screening hit compounds on the activity of human telomerase in a cell-free assay, we performed the telomere repeat amplification protocol (TRAP) assay [3] by using crude cellular extracts from A431 human tumor cells as a source of telomerase. Chrolactomycin exhibited potent inhibition of human telomerase in a dose-dependent manner with an IC_{50} of 0.5 μM (Figure 3A). UCS1025A also exhibited potent inhibition of human telomerase with an IC_{50} of 1.3 μM, but radicicol did not exhibit direct inhibition of human telomerase in TRAP assay (data not shown). These results demonstrated that the selective inhibition by chrolactomycin or UCS1025A of growth of the telomere-shortened yeast strain was due to direct inhibition of telomerase activity in yeast. In this report, we focus on the study of the effects of chrolactomycin on human telomerase. Details of the mechanism of action of UCS1025A and radicicol will be reported elsewhere.

Telomerase Inhibition in Human Cancer Cells by Chrolactomycin

We next examined whether chrolactomycin was capable of inhibiting telomerase activity in human tumor cells. To examine the long-term effects of chrolactomycin on telomerase-positive human renal carcinoma ACHN cells, we first determined the drug concentration at which cellular telomerase could be inhibited without acute growth-inhibitory effects. Chrolactomycin had only minor effect on short-term cell viability and proliferation, as determined in a 4 day cytotoxicity assay with concentrations
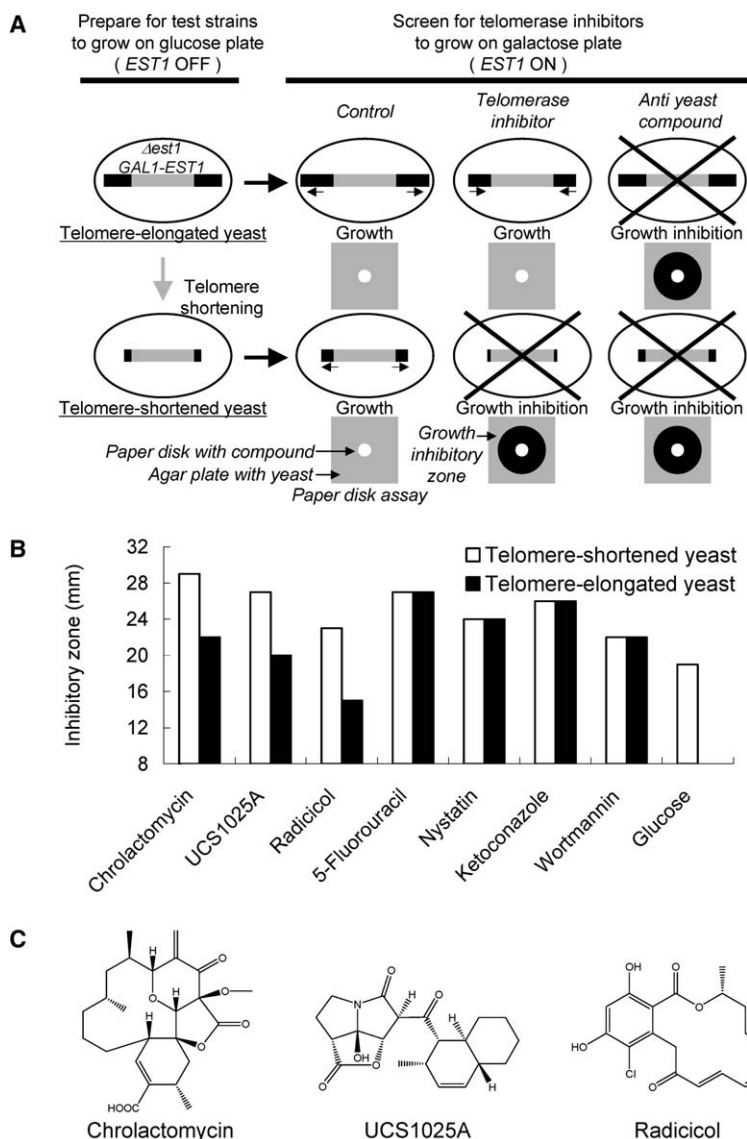
Figure 2. Selective Growth Defects in Telomere-Shortened Yeast by Chrolactomycin, UCS1025A, and Radicicol

(A) The principle of the yeast-based assay to identify telomerase inhibitors.

(B) Sterile filter disks (diameter is 8 mm) impregnated with 15 nmol chrolactomycin (telomerase inhibitor), 30 nmol UCS1025A (telomerase inhibitor), 50 nmol radicicol (telomerase inhibitor/Hsp90 antagonist), 10 nmol 5-fluorouracil (pyrimidine antagonist), 20 nmol nystatin (cell membrane sterol binder), 20 nmol ketoconazole (ergosterol synthesis inhibitor), 200 nmol wortmannin (phosphatidylinositol 3-kinase inhibitor), or 22 μmol glucose (positive control to shut off *EST1* expression) were placed on two agar plates, containing telomere-shortened or -elongated yeast strains, simultaneously. The diameters of the growth-inhibitory zone surrounding the paper disk were measured after 2 days of incubation at 30°C. Representative results of three similar experiments are shown.

(C) Chemical structures of chrolactomycin, UCS1025A, and radicicol.

of 1 or 2 μM, the same level as the IC$_{50}$ value for inhibition of telomerase in a cell-free assay. ACHN cells were cultured with chrolactomycin at 1 or 2 μM for 7 days. At this time point, telomerase activity in cell extract was reduced by 76% at 1 μM and by 85% at 2 μM (Figures 3B and 3C). Similar results were obtained with other human cancer cells, such as renal carcinoma Caki-1 and colorectal adenocarcinoma WiDr, treated with chrolactomycin (data not shown). On the basis of these observations, the concentration of chrolactomycin for use in long-term culture was determined to be 1 or 2 μM.

Effects on Telomere Length and Cell Growth of Human Cancer Cells after Long-Term Treatment with Chrolactomycin
During long-term culture, ACHN cells were monitored periodically by telomere length estimation by using Southern blot analysis (Figure 4A). Control cells treated with DMSO alone exhibited a heterogeneous size distribution, with an average telomere length of ~2.3 kb (Figure 4B). TRF length was progressively shortened as cells were propagated in the presence of chrolacto-

mycin. The telomere erosion rates between day 7 and day 39 were calculated to be 17 and 40 bp/PD at 1 and 2 μM, respectively (Figure 4D). In contrast, DMSO-treated cells maintained a stable TRF length. These telomere erosion rates are slightly lower than the speed of telomere erosion reported in the absence of telomerase (25–200 bp/PD), which is largely due to the end replication problem [19–21].

We characterized the growth properties of chrolactomycin-treated cells (Figure 4C). ACHN cells in the absence or presence of 1 or 2 μM chrolactomycin initially exhibited only minor differences in proliferation. After 60 days, however, 2 μM chrolactomycin-treated ACHN cells exhibited nearly complete inhibition of proliferation. One μM chrolactomycin-treated cells exhibited a delay of proliferation.

Cell Cycle Distribution and Senescence-Associated β-Galactosidase Staining in Human Cancer Cells Treated with Chrolactomycin
Analysis of cell cycle distribution of chrolactomycin-treated ACHN cells for 46 days was performed by
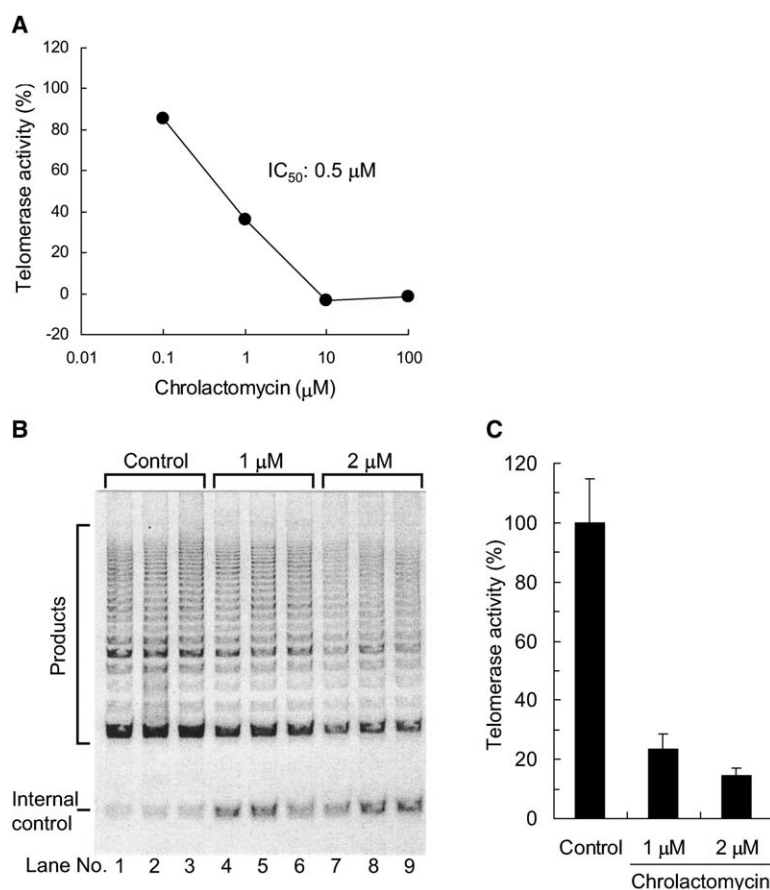
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Figure 3. Inhibition of Human Telomerase by Chrolactomycin in a Cell-free Assay and in a Cellular Assay

(A) Chrolactomycin was incubated with the extracts from human epidermoid carcinoma A431 cells for 30 min. An aliquot of the mixture was subjected to TRAP analysis by using the $TRAP_{EZE}$ XL telomerase detection kit for measurement of telomerase activity. Values are the means (n = 2).

(B) Human renal carcinoma ACHN cells were preincubated for 24 hr at 37°C and were then treated with chrolactomycin at 1 or 2 μM for 7 days. After washing with PBS, cell lysate was prepared with lysis buffer. Extracts with equivalent protein concentrations were prepared and subjected to TRAP analysis with the $TRAP_{EZE}$ telomerase detection kit for measurement of telomerase activity.

(C) Percentages of inhibition of telomerase in ACHN cells by chrolactomycin at 1 and 2 μM were 76% and 85%, respectively, as determined by scanning the gel described in Figure 3B. Values are the means ± SD (n = 3).

propidium iodide signal staining with flow cytometry (Figure 5A). Chrolactomycin at 1 μM increased the population of cells in G2/M phase and decreased that in G1 phase. Chrolactomycin at 2 μM increased the population of cells in the G2/M phase, the population of cells in the aneuploidy fraction, and the population of cells in the sub-G1 fraction.

Chrolactomycin-treated, late-passage ACHN cells exhibited distinctive morphological features associated with cellular senescence. In contrast to DMSO-treated cells, ACHN cells treated with 2 μM chrolactomycin at day 50 exhibited a flattened morphology and increased size, contained multiple nuclei, and exhibited induction of senescence-associated β-galactosidase activity (Figure 5B). These results, in conjunction with those for cell cycle analysis, indicate that chrolactomycin induced cellular senescence in human tumor cells as a major effect of long-term treatment, consistent with previously reported cellular effects of telomerase inhibition [22–24].

Discussion

Yeast-Based Screening Focused on Replicative Senescence Is Useful for Identifying Direct or Indirect Inhibitors of Human Telomerase

The focus of our research has been to identify novel telomerase inhibitors. A number of small-molecule telomerase inhibitors have been reported. Most inhibitors have been identified by random biochemical screening or by design of antisense oligonucleotides. Unlike cell-free biochemical assays, cell-based assays have been shown to have several advantages, such as an increased efficiency in selection of cell-permeable small molecules and an ability to identify the small molecules with several modes of action. To accomplish our objective, we established a forward chemical genetics approach and used a strategy different from those previously applied to screening for telomerase inhibitors. We focused on replicative senescence caused by telomere erosion in the absence of telomerase due to the end replication problem. Two sets of yeast strains that differ only in telomere length were prepared by regulating the expression of the *EST1* gene. One of the test strains was a telomere-shortened yeast strain, like human cancer cells with short telomere length and active telomerases, and another was a telomere-elongated yeast strain, like human stem cells with long telomere length and active telomerases [3–5]. Since the telomere-shortened yeast strain rapidly entered cell senescence and exhibited a growth-defective phenotype in the absence of active telomerase, compounds that induced selective growth defects in this telomere-shortened yeast strain could represent new drug candidates acting on telomeres and telomerase.

Consistent with this expectation, screening with this yeast-based assay allowed us to identify three structurally unrelated natural products, chrolactomycin, UCS1025A, and radicicol, as inhibitors active against human telomerase. TRAP assay with human telomerase revealed that chrolactomycin and UCS1025A directly inhibited telomerase activity in in vitro enzyme assays, while radicicol did not. At the cellular level, however, all
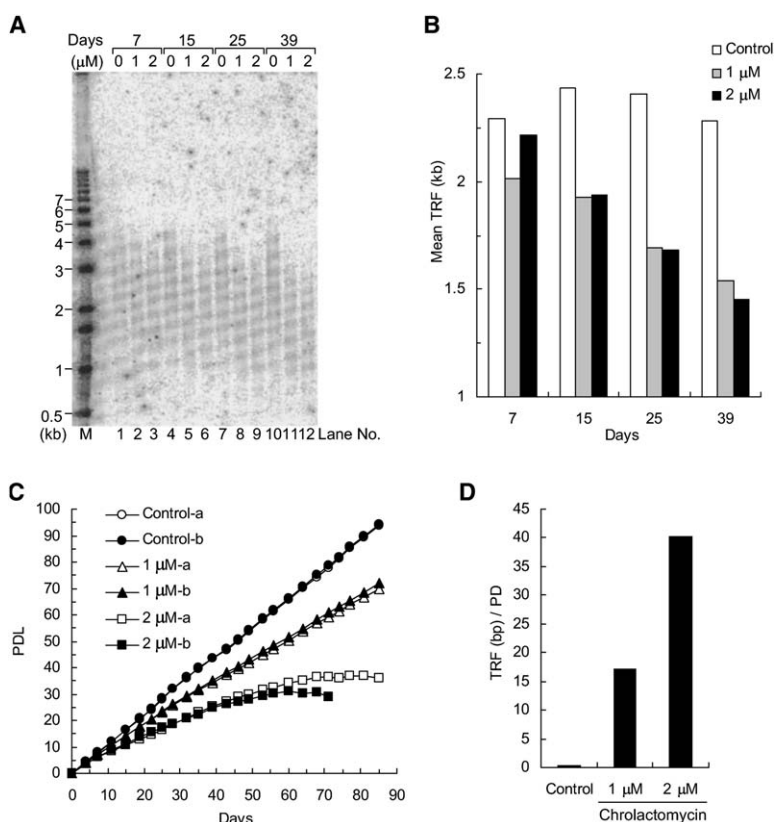
Figure 4. Effects of Chrolactomycin on Telomere Length and Cell Proliferation of ACHN Cells during Long-Term Exposure

(A) Measurement of TRF in ACHN cells treated with chrolactomycin. Genomic DNA isolated from ACHN cells treated with chrolactomycin was digested and subjected to Southern blot analysis.

(B) Mean TRF lengths calculated from size distributions in scanned lanes.

(C) Dose-dependent inhibition of ACHN cell proliferation by prolonged treatment with 1 or 2 μM chrolactomycin. Results of two parallel experiments are shown.

(D) The rates of telomere loss between day 7 and day 39 were calculated to be 17 and 40 bp/PD with 1 and 2 μM chrolactomycin treatment, respectively.

three compounds exhibited inhibitory effects against telomerase in human cancer cells, indicating that they can be classified into two groups of new telomerase inhibitors: direct inhibitors (chrolactomycin and UCS1025A) and an indirect inhibitor (radicicol) (see below).

Telomerase activity has been characterized for a wide range of organisms, and genes encoding both the RNA and protein components of the enzyme complex have been identified [25]. The catalytic subunit human telomerase reverse transcriptase (TERT) was identified as a human homolog of p123 protein in *Euplotes aediculatus* and of Est2p in *S. cerevisiae* [26]. According to recent studies, the telomerase-associated factor Est1p in *S. cerevisiae* is evolutionarily conserved in humans [27]. Based on these similarities between yeast and human telomerase components, it could be considered appropriate that the active compounds found by the yeast-based assay would exhibit an inhibition of human telomerase activity through effects on phylogenetically conserved portions of telomerase components.

Radicicol was originally isolated as an antifungal antibiotic and has been recently rediscovered with its unique mode of action as an Hsp90 antagonist in our laboratories [11, 12]. Hsp90 and p23, the molecular chaperones, are required for the assembly of active telomerase holoenzyme by binding to the catalytic subunit of telomerase [28]. Geldanamycin, another Hsp90 antagonist, blocks assembly of active telomerase in cells. Therefore, radicicol is also thought to affect telomerase activity through interaction with Hsp90.

Thus, identification of a new chemical class of telomerase inhibitors demonstrated the utility of the yeast-based assay used in this study for discovery of small-molecule inhibitors of human telomerase.

Chrolactomycin Is a Lead Compound as a Novel Telomerase Inhibitor

Chrolactomycin was a novel microbial metabolite isolated in our laboratories, but its biological activity had not been investigated sufficiently. In addition to the telomerase inhibition detected with an in vitro enzyme assay, consistent results of analyses with several human cancer cell lines confirm that chrolactomycin exhibits telomerase inhibitory activity at the cellular level. Long-term culture of ACHN cells with chrolactomycin revealed population-doubling-dependent antiproliferative activity accompanied by telomere shortening. At late passage, chrolactomycin-treated ACHN cells exhibited distinctive features associated with cellular senescence and an increase in the G2/M and aneuploid fractions. Similar results have been previously reported in cases of inhibition of telomerase by epigallocatechin gallate [22], BIBR1532/BIBR1591 [23], TNQX [24], and hTERT-shRNA [29]. These results suggest that chrolactomycin meets the criteria for a telomerase inhibitor and may be useful for the development of new antitumor agents acting on human telomerase.

Structural studies of chrolactomycin showed that the exomethylene group is the structural feature responsible for inhibition of telomerase. The exomethylene group can react with a biological nucleophile, especially the sulfhydryl group of a cysteine residue, by a Michael-type reaction. Among known telomerase inhibitors, the isothiazolone moiety of TMPI, a telomerase inhibitor,
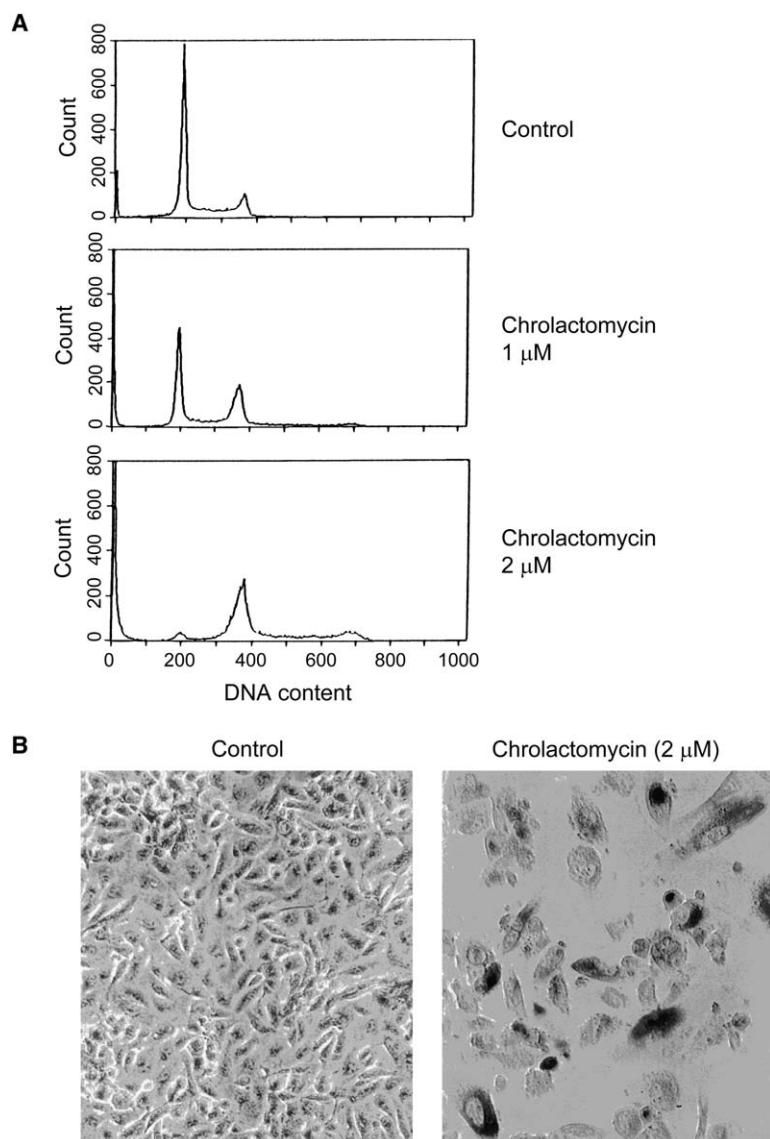
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was reported to interact potentially with the sulfhydryl group of one or more cysteine residues at or near the active site of telomerase [30]. The CP motif in the N-terminal region of TERT included an evolutionarily conserved cysteine residue that has been demonstrated to be required for binding of TR [31, 32]. Therefore, chrolactomycin might covalently bind to the cysteine residue in the CP motif of TERT through a Michael addition reaction with the exomethylene group, resulting in inhibition of telomerase activity. In addition, as an example of an inhibitor with a similar mode of action, PD 168393, a Michael acceptor-containing inhibitor, was designed to covalently modify an active site cysteine of ErbB family tyrosine kinase [33]. The irreversibility of PD 168393 has been demonstrated to confer a significant therapeutic advantage over equally potent reversible analogs in terms of in vivo tumor suppression. Considering these unique features of the mode of action of chrolactomycin, we are currently proceeding to structure-activity relationship studies to optimize its efficacy and evaluation of the antitumor activity of chrolactomycin in in vivo mouse models.

Significance

Cell-based screening have been shown to have several advantages, such as an increased efficiency in selection of cell-permeable small molecules and an ability to identify the small molecules with several modes of action. Since the telomere-shortened yeast strain rapidly entered cell senescence and exhibited a growth-defective phenotype in the absence of active telomerase, we therefore attempted to develop a new assay system for drug candidates acting on telomeres and telomerase by applying yeast genetics. Consistent with this expectation, screening with this yeast-based assay allowed us to identify three structurally diversified natural products, chrolactomycin, UCS1025A, and radicicol. Chrolactomycin and UCS1025A were novel microbial metabolites produced by *Streptomyces* sp. and *Acremonium* sp. and were isolated in our laboratories, but their biological activities had not been investigated sufficiently. Inhibitory studies against human telomerase from human cancer cells in cell-free and cellular systems indicated that active compounds

could be classified into two groups of new telomerase inhibitors: direct inhibitors (chrolactomycin and UCS1025A) and an indirect inhibitor via inhibiting Hsp90 function (radicicol). Thus, identification of new telomerase inhibitors demonstrated the utility of the yeast-based assay used in this study for discovery of small-molecule inhibitors of human telomerase. Furthermore, long-term culture of cancer cells with chrolactomycin revealed population-doubling-dependent antiproliferative activity accompanied by telomere shortening and expression of a senescence-associated phenotype. Therefore, chrolactomycin, which meets the criteria for a telomerase inhibitor, might be a useful lead for the development of new antitumor agents acting on human telomerase.

Experimental Procedures

Chemicals
Chrolactomycin, UCS1025A, radicicol, and wortmannin were produced by fermentation and were purified in our laboratories. Nystatin, 5-fluorouracil, and ketoconazole were purchased from Sigma.

Yeast Strains and Plasmids
Yeast strains were maintained by using standard media and growth conditions. Yeast strains used in this study were derived from *Saccharomyces cerevisiae* NBW6 (*MATa pho3-1 leu2 ura3 his3 trp1 ade2 can1*). The *EST1* gene was cloned by PCR by using yeast genomic DNA as a template. KY19504 (*MATa pho3-1 leu2 ura3 his3 trp1 ade2 can1 ΔEST1::URA3*) was produced by one-step gene replacement of *EST1*. *EST1* disruption was confirmed by Southern blot analysis. The plasmid pHK209 expressing the *EST1* gene controlled by the *GAL1* promoter is a YCp-based plasmid containing the *LUE2* selectable marker. KY19614 (*MATa pho3-1 leu2 ura3 his3 trp1 ade2 can1 ΔEST1::URA3 [LEU2 GAL1-EST1]*) was constructed by transformation of KY19504 with pHK209.

Screening System for Telomerase Inhibitors in Yeast
The level of expression of *EST1* could be controlled by the carbon source of media for *S. cerevisiae* KY19614. After this yeast was subcultured 7–9 times on agar medium containing glucose as a carbon source, it entered cell senescence and exhibited a growth-defective phenotype accompanied by telomere shortening. Therefore, cells harvested just before they entered cell senescence were used as a test strain, and they were named the telomere-shortened yeast strain. Two kinds of yeast cells with different telomere lengths were used for assay. One of the test strains was a telomere-shortened yeast strain, and another was a telomere-elongated yeast strain. Both were grown in synthetic medium containing 20 g/l galactose, 2 g/l sucrose, 7 g/l yeast nitrogen based without amino acid, 50 mg/l histidine, 50 mg/l tryptophan, and 50 mg/l adenine.

Cell Culture
Human renal adenocarcinoma ACHN cells were purchased from American Type Culture Collection. Cells were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Cells were culture in MEM supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM nonessential amino acids.

Measurement of Telomerase Activity
Telomerase activity was measured by a TRAP assay with the TRAP$_{EZE}$ XL telomerase detection kit (Intergen), which is a quantitative fluorescent-labeled PCR system for the estimation of relative telomerase activity with the use of a PCR internal control. To prepare cell extracts for telomerase assays, human epidermoid carcinoma A431 cells were lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamide, 0.5% 3-([3-cholamidopropyl]-dimethyl-ammonio)-1-propane sulfonate (CHAPS), and 10% glycerol. To evaluate the inhibitory activity of a test compound under appropriate conditions, a titration experiment was performed to determine an optimal amount of cell extract. Test compounds

were incubated with the extracts for 30 min at 30ºC. The intensities of the PCR products were measured by using an ARVOsx 1420 multilabel counter (Wallac) to detect the levels of fluorescein and sulphorhodamine with the appropriate excitation and emission filters. The levels of telomerase activity were quantified by the ratio of the fluorescein intensity of the entire TRAP ladder to the sulphorhodamine intensity of the internal control after the correction of each fluorescent intensity for the negative control and the background.

Cell-Based Telomerase Assay
The cells were treated with different dilutions of test compounds for 7 days at 37ºC in a humidified atmosphere with 5% CO_2. After washing with PBS, cell lysate was prepared with lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamide, 5 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol). Extracts with equivalent protein concentration were prepared and subjected to TRAP analysis by using the TRAP$_{EZE}$ telomerase detection kit (Intergen) for measurement of telomerase activity. After extension of the substrate by telomerase, the products were amplified by PCR, resolved in 12.5% polyacrylamide gels, and revealed by staining with SYBR Green I (Molecular Probes) and scanning with a FluorImager SI (Molecular Dynamics). Two distinct areas were evaluated separately: the telomerase activity-related ladder bands and the 36 bp internal control band. The signals of the telomerase ladders of the lanes were normalized to the intensities of the internal controls.

Long-Term Exposure Studies
Cells were grown in six-well plates at 1.1×10^5 cells/well. After pre-incubation for 4–5 hr, cells were exposed to a nonacute cytotoxic concentration of test compounds or an equivalent concentration of DMSO (vehicle control) every 24 hr. Every 3–4 days, the cells in control and drug-treated wells were trypsinized, counted, and re-seeded. The remaining cells were collected and used for measurements of telomerase activity, telomere length, and cell cycle distribution.

Cell Cycle Analysis
Cells were harvested by trypsin-EDTA treatment, fixed with 70% ice-cold ethanol, and stored at 4ºC. For flow cytometric analysis, the RNA was hydrolyzed with 250 μg/ml RNase A (type 1-A; Sigma) at 37ºC for 30 min, and the cells were stained with propidium iodide (Sigma) for 20 min. The DNA content of cells was analyzed with an EPICS ELITE flow cytometer (Coulter Electronics).

Human TRF Length Analysis
Human genomic DNA was extracted with DNAzol (GIBCO-BRL). A total of 5 mg DNA was digested with *HinfI/RsaI* and separated by 0.5% agarose gel electrophoresis. The gels were dried with a gel dryer, denatured (0.5 M NaOH, 1.5 M NaCl) at room temperature for 30 min, and neutralized (0.5 M Tris-HCl [pH 7.0], 1.5 M NaCl) for 15 min. Gels were prehybridized in hybridization buffer (0.5 M Na_2HPO_4, 1 mM EDTA [pH 7.2], 1% BSA, and 7% SDS) at 37ºC for 1 hr. Gels were then hybridized with the end-labeled, single-stranded telomeric oligonucleotide, $(TTAGGG)_3$, probe prepared with a DNA 5′ end labeling kit (MEGALABEL, TaKaRa) at 37ºC overnight, and they were washed with 0.5× SSC for 10 min twice and dried with a gel dryer for 30 min. To determine TRF length, a BAS2000 (Fuji Photo Film) was used to quantitate the strength of the radioactive signal in each lane.

Senescence-Associated β-Galactosidase Staining
Cells were washed in PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS at room temperature, washed in PBS, and incubated for 16 hr in staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside [X-Gal], 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$).

Supplemental Data
Supplemental Data including information on telomere shortening by radicicol in yeast are available at http://www.chembiol.com/cgi/content/full/13/2/183/DC1/.

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